Original Article

Immunosuppression, peripheral inflammation and invasive infection from endogenous gut microbiota activate retinal microglia in mouse models

Running title: Factors affecting retinal microglia

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ABSTRACT

Although its actual role in the progression of degenerative processes is not fully known, the persistent activated state of retinal microglia and the concurrent secretion of inflammatory mediators may contribute to neuronal death and permanent vision loss. Our objective was to determine whether non-ocular conditions (immunosuppression and peripheral inflammation) could lead to activation of retinal microglia. Mouse models of immunosuppression induced by cyclophosphamide and/or peripheral inflammation by chemically-induced sublethal colitis in C57BL/6J mice were used. Retinal microglia morphology, spatial distribution and complexity, as well as MHCII and CD11b expression levels were determined by flow cytometry and confocal immunofluorescence analysis with anti-CD11b, anti-IBA1 and anti-MHCIIRT1B antibodies. The retinas of mice with double treatment showed changes in the microglial morphology, spatial distribution and expression levels of CD11b and MHCII. These effects were higher than those observed with any treatment separately. In addition, we also observed in these mice (i) translocation of endogenous bacteria from gut to liver, and (ii) upregulation of TLR2 expression in retinal microglia. Using a mouse model of immunosuppression and gut colonization by *C. albicans*, translocation of fungal cells was confirmed to occur in wild type and, to a higher extent in TLR2 KO mice, which are more susceptible to fungal invasion; interestingly microglial changes were also higher in TLR2 KO mice. Hence, non-ocular injuries (immunosuppression, peripheral inflammation and invasive infection from endogenous gut microbiota) can activate retinal microglia and therefore could affect the progression of neurodegenerative disorders and should be taken into account to improve therapeutic options.

Key words: colitis; fungal colonization; immunosuppression; retinal microglia

1. Introduction.

In ocular neurodegenerative disorders, such as diabetic retinopathy or glaucoma, a highly intricate situation takes place involving a plethora of cytokines and other factors, some of them harmful and other protective, ending in a biased effect towards neuronal death and permanent vision loss. In order to improve therapeutic approaches we need to better understand all the factors involved in the progression of the disease (1). It is now accepted that an excessive or prolonged activation of microglial cells, both in the brain and retina, can be one of the primary factors that may lead to chronic inflammation and irreversible neuronal death in degenerative disorders (1-3). Microglial cells act as a phagocytotic cell population, with a relevant role in both physiological and pathological conditions. Several reports point that microglia are important for photoreceptor survival in retinal dystrophies (3-6). In a resting state microglia participate in the clearance of damaged cell debris from the inner retinal layer and secrete neuroprotective factors that protect photoreceptors via Müller glia. When an infection or another harmful stimulus that challenges the homeostatic state occurs, microglial cells turn into an activated state, displaying a variety of distinct functional phenotypes, and showing an increase in the expression of several surface markers, such as major histocompatibility complex class II (MHCII) molecules, which are expressed only in activated microglial cells (7). In this activated state microglial cells can proliferate, migrate to the site of the stimulus, show greater phagocytic capacity, and secrete inflammatory mediators, although their protective or harmful role still remains controversial (1-3).

Systemic infections (8), immunosuppression (9, 10), and peripheral inflammation (11, 12), can increase microglia activation in the brain, and there is recent evidence suggesting a relationship between persistent immune activation and neurodegenerative

disorders (13). Previous reports have demonstrated that systemic infection by fungus or virus can also activate the retinal microglia (14, 15), possibly constituting a risk factor for patients with retinal neurodegenerative diseases. Therefore, in a similar way, immunosuppression and peripheral inflammation might also be considered as risk factors for those patients. It should also be considered that most disseminated infections come from an endogenous origin, involving the translocation of the pathogen across the gut mucosa to the bloodstream (16-21), a process that is favoured by immunosuppression, gut colonization and inflammatory processes affecting gut mucosa (22-25).

Therefore, the aim of this work was to determine whether immunosuppression and GI inflammation, which may be correlated to a systemic infection from endogenous origin, could induce retinal microglia activation and hence should be taken into account as risk factors in the progression of degenerative diseases.

2. Materials and Methods

Animals

This study was carried out in strict accordance with the recommendations of the "Royal Decree 1201/2005, BOE 252" for the Care and Use of Laboratory Animals, of the "Ministry for the Presidency", Spain. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Valencia (Permit Number: A1264596506468). All animals were handled in accordance with current regulations for the use of laboratory animals (NIH, ARVO and European Directive 2010/63/UE) in order to minimize animal suffering and limit the numbers used for the experiments. Female C57BL/6J mice (8-10 weeks old) purchased from Harlan Ibérica (Barcelona, Spain) and TLR2 knockout mice (C57BL/6 background) kindly provided

by Dr. S. Akira (University of Osaka, Japan) were bred and maintained under specific pathogen-free conditions at the University of Valencia animal facilities.

Mouse models.

Mice were given two oral doses of 100 mg/Kg of cyclophosphamide (CPA) in 100 ml of PBS on days 1 and 3. The retinas were analyzed 5 days after the end of the treatment (day 8). To induce sub-lethal colitis, mice were given 1% (w/v) dextran sulphate sodium salt (DSS) (36,000 - 50,000 kDa; MP Biomedicals Europe, Illkirch, France) in drinking water *ad libitum*, for 5 days (26). For mice receiving a combination of DSS plus CPA, the latter was administered on days 3 and 5 after the beginning of the treatment with DSS. Untreated mice were used as controls. Mice were sacrificed by cervical dislocation. Retinas were analyzed 5 days after the end of the treatment (day 10), and the bacterial burden in liver was quantified by CFU determination on Mueller-Hinton plates according to standard microbiological methods.

Sustained high level of gut colonization with *Candida albicans* CAF2 strain was performed according to previously described methods (25, 27). Briefly, mice were treated with antibacterial antibiotics four days (day -4) prior to yeasts administration (day 0), and maintained during the assays; the antibiotic treatment was administered in sterile drinking water *ad libitum* containing 2 mg/ml streptomycin (Sigma), 1 mg/ml bacitracin (Sigma) and 0.1 mg/ml gentamicin (Sigma). Colonization with *C. albicans* CAF2 strain was performed (day 0) by a single gavage of 10^7 yeast cells in 100 µl of sterile PBS. Translocation of fungal cells from GI-tract to internal organs was achieved in colonized mice following administration of three oral doses of CPA, as indicated above, on days 6 and 4 and 2 prior to sacrifice by cervical dislocation, as

described elsewhere (28). Fungal burden in internal organs (liver and kidney) was quantified by colony forming units (CFUs) determination on Saboureaud-dextrose agar plates added with chloramphenicol and gentamicin, following standard procedures previously described (26, 29).

Flow cytometry analysis.

Mice were sacrificed by cervical dislocation. The eyes were enucleated and the retinas were carefully dissected. Disaggregated cells were labeled with FITC-conjugated anti-CD11b antibody (Clone M1/70, eBioscience, San Diego, CA) or its control isotype, according to standard procedures. In some assays disaggregated retina cells were also labelled with PE-conjugated anti-TLR2 antibody (clone 6C2, eBioscience, San Diego, CA) or its control isotype. Flow cytometry analyses were performed in a FACSCanto cytometer (BD Biosciences) and the data were analyzed with FACSDiva software. The areas in dot plots were measured as previously described (14). Experiments were performed, at least, with three animals. Results are expressed as mean ± SD and $*P<0.05$, $**P<0.01$, $***P<0.001$ values were considered significant.

Immunohistochemical analysis.

Analysis of retinal slices was performed as previously described (14). Briefly, retinal cryosections were incubated with a cocktail of mouse anti-MHC class-II RT1B monoclonal antibody, 1:50 (clone OX-6, AbD Serotec, Kidlington, United Kingdom) and rabbit anti-IBA1 polyclonal antibody, 1:500 (Wako Chemicals, Neuss, Germany). For the study of Müller gliosis a rabbit anti-GFAP antibody, 1:200 (Dako, Denmark A/S) was used. Secondary antibodies Alexa Fluor 555-conjugated donkey anti-mouse

IgG (Molecular Probes, Eugene, OR) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG were used, both at a 1:100 dilution. Samples were visualized and photographed under a laser scanning confocal microscope (TCS SP2 Leica Microsystems, Wetzlar, Germany). Immunohistochemical negative controls omitting the primary antibodies were included. As positive control for degeneration and gliosis we used retinal sections of rd10 mice, a model of autosomal recessive retinitis pigmentosa (30).

3. Results

Retinal microglia changes in mouse models of immunosuppression and inflammation.

Retinal microglia changes in immunosuppressed mice with or without concomitant chemically induced colitis, were first observed as an increase in the heterogeneity of the CD11b positive population in FSC-CD11b-FITC dot and density plots, as previously described (14) (Fig. 1A-E). In control mice, CD11b positive cells appeared as a homogeneously sized population (Fig. 1A,B), similar to CD11b positive cells from DSS-treated mice (Fig. 1C), whereas in both, CPA-treated (Fig. 1D) and DSS+CPA-treated mice (Fig. 1E) increased heterogeneity was observed. Accordingly, the area value of the delimited CD11b positive population was significantly higher in CPA-treated animals (157 %) compared to control (100 %). This difference was higher in the case of simultaneous treatment with DSS (176 %). The area value of DSS-treated animals showed no significant differences (102 %) (Fig 1F). We also observed differences in the mean FITC-CD11b fluorescence values of control (100 %), DSS-treated (111 %), CPA-treated (132 %) and DSS+CPA-treated groups (164 %) (Fig 1G). Labelling with control isotype antibodies resulted in a non-specific background signal

similar in all cases (not shown). CD11b positive cells constituted 0.07 % of the total population of control retinas and a slight but significant increase was observed in all treated groups (about 0.11 %) without significant differences among them (Fig. 1H).

Microglia changes in morphology and spatial distribution was demonstrated by immunostaining of retinal sections (Fig. 2). In control animals, Iba1 positive cells (the entire microglial population) showed the usual morphology of a resting state -small cells with rounded soma and various branching processes-and were mainly located at the inner plexiform layer (Fig. 2A). These microglia showed no reactivity against the activation marker MHCII (Fig. 2 B, C). In CPA- and DSS+CPA-treated animals, Iba1 positive cells showed an elongated soma (Fig. 2D), reactivity against MHCII (Fig. 2 E,F) and were located in the outer plexiform, inner nuclear, inner plexiform and ganglion cell layers (Fig. 2 D-F). No labelling was observed when the primary antibodies were omitted (not shown), and staining with control isotype antibody resulted in a non-specific background signal similar in retinal microglia from both control and treated mice (not shown).

Retinal staining with hematoxylin (Fig. 3A-C) showed that the retinal structure was intact in DDS+CPA treated mice (Fig. 3B) and no differences were observed between these and control (Fig. 3A), in contrast to its appearance in an animal model of retinitis pigmentosa, the rd10 mouse, which has an altered retinal organization with evident thinning of the outer nuclear layer (Fig. 3C). In agreement, immunohistochemical analysis of retinal sections stained with anti-GFAP antibody showed no Müller cell gliosis in control (Fig. 3D) or DSS+CPA-treated mice (Fig. 3E). As a positive control of a gliotic state, we analyzed the retinas of rd10 mice (Fig. 3F),

given that Müller cell gliosis is a common phenomenon in mice carrying this mutation (31, 32).

Gut microbial translocation in murine models of immunosuppression.

In the case of immunosuppression and peripheral inflammation (DSS-CPA treated mice), bacterial translocation of the gut microbiota to the liver was detected. Bacterial burden in liver of these DSS-CPA treated mice showed a high variability among animals (ranging from $1x10^3$ to $35x10^3$ UFC/g, with and average value of $13x10^3$), whereas bacterial burden in CPA- or DSS-chemically treated mice was not detected.

To further check the relationship between infection from endogenous gut microbiota and retinal microglia changes, we performed assays with control and TLR2 KO mice colonized with *C. albicans*, since TLR2 KO mice are more susceptible to infections by this species (29, 33). Mice were immunosuppressed and treated with antibacterial antibiotics to favour disseminated candidiasis (24, 25, 27). In these conditions, we found that TLR2 KO mice showed significant higher levels (about 4-fold) of fungal infection in internal organs (average values: 2214 UFC/g liver and 4297 UFC/g kidney) than the wild type (average values: 369 UFC/g liver and 2214 UFC/g kidney), as previously described (28). As expected, in our experimental conditions no bacterial presence was detected in these organs.

The CD11b positive population of *C.albicans* colonized mice showed an increased heterogeneity in FSC-CD11b-FITC dot and density plots (not shown). The area value of the delimited microglia population was higher (about 10%), although not statistically significant, in treated TLR2 KO mice than in treated wild type animals (Fig 4A). The FITC-CD11b fluorescence mean values were significantly increased (about

30%) in TLR2 KO mice as compared to the wild type (Fig. 4B). Labelling with control isotype antibodies resulted in a non-specific background signal similar in all cases (not shown). No differences were found in retinal microglia percentage (0.07%) in wild type or TLR2 KO treated animals (Fig. 4C), similar to untreated controls, probably due to an increased immunosuppressive effect of the CPA treatment used.

TLR2 is upregulatead in retinal microglia of immunosuppressed mice with peripheral inflammation.

We have previously described that TLR2, a pathogen recognition receptor, is expressed in murine retinal microglia (34). Therefore, as DSS-CPA treatments cause retinal microglia activation and bacterial translocation from GI-tract to internal organs, we wonder whether this may also lead to changes in TLR2 expression. As shown in Fig. 5, retinal microglia cells (CD11b+ population) from DSS-CPA treated mice showed an increased expression of TLR2, as compared to control mice, whereas no differences between both groups were found following labelling with control isotype antibodies (Fig. 5). This result indicates that expression of TLR2 is upregulated (about 50% increase) in DSS-CPA treated mice, further supporting that retinal microglia is activated in these conditions.

4. Discussion

To date, retinal neurodegenerative disorders unavoidably drive to permanent vision loss. Recent evidence suggests that patients affected with these pathologies could be benefited by the use of therapeutic agents that preserve retinal physiology for a more extended period of time (1). Since one of the factors that are likely involved in retinal degenerative processes is a chronic or excessive activated state of the retinal microglia,

we tried to identify non-ocular conditions that could induce or enhance its activation and thus affect the progress of neurodegenerative disorders, with the hope that this knowledge could lead to more appropriate therapeutic approaches.

Our data indicate that CPA, a widely used chemotherapeutic agent, may cause changes in retinal microglia morphology and location, as well as an increase in the expression of different markers, which is in accordance with previous results in the brain (9, 10). Probably this is not due to a direct effect of CPA on retinal cells, as CPA does not cause ocular toxicity itself. In fact, it can improve ocular manifestations and can help to maintain visual acuity in patients with immune system-related diseases (35-37). However, we cannot rule out the possibility that endogenous self-components generated by the killing effect of CPA on proliferative cells could be ultimately responsible of the observed changes. In the case of immunosuppression and peripheral inflammation, bacterial translocation could provide a good explanation for microglial changes in the retina, since bacterial translocation of the gut microbiota to the liver was detected. It must be considered that microbial-derived ligands can induce or enhance microglia activation following a direct recognition through pattern recognition receptors (34). Recent results also suggest a cross-talk between gut microbiota and bone marrow, as microbiota-mediated signalling promotes the hematopoietic differentiation of myeloid cells in healthy individuals and microbiota-driven myelopoiesis requires recognition of microbiota-derived products by TLRs (38). Therefore, the possibility that microbial ligands from GI microbiota could induce retinal microglia changes in CPA-treated mice should be also considered. This suggests that steady state of microglia activation could be the result of the recognition of soluble microbial ligands and, therefore, that the microbiome may play a role in modulating microglia even in healthy individuals.

In our model, the treatment with DSS+CPA also increased (roughly 50%) the expression of TLR2 by microglial cells (not shown), suggesting that the up regulation of both MHCII and TLR2 expression probably improves the ability of activated microglia to deal with pathogens, by increasing both, the antigen presenting and microbial recognition capacities. Several factors may be involved in TLR2 upregulation, such as response to inflammatory cytokines and/or recognition of microbial ligands by retinal microglia. In addition, we propose upregulation of TLR2 to be considered as a candidate marker for retinal microglia activation.

The notion that systemic infection from endogenous gut microorganisms could induce or enhance retinal microglia activation is further supported by the results obtained with immunosuppressed and *C. albicans* colonized mice. In TLR2 KO mice we also observed an increased heterogeneity of retinal microglia as compared to wild type treated mice, which may be due to the higher levels of fungal burden in these mice. However, other issues should also be considered. As mentioned above, CPA treatment itself may cause changes in retinal microglia, and may differentially affect to the gut barrier. In addition, retinal microglia activation can be mediated (i) by proinflammatory responses to infection, although production of proinflammatory cytokines in response to *C. albicans* is strongly dependent on TLR2 recognition (29, 33), and (ii) by direct recognition of the pathogen or pathogen-derived ligands, a process that involves TLR2 as well as other host pattern recognition receptors, such as TLRs other than TLR2, dectin-1, dectin-2, dectin-3, galectin 3 and mannose receptors, among others (33, 39, 40). Furthermore, from our results we conclude that TLR2 appears to be dispensable for retinal microglia activation upon invasive fungal infection. In a previous work we showed that, in the absence of immunosuppressive treatment, disseminated candidiasis

following intravenous injection of yeast caused a 6-fold increase in microglia population compared to non-infected mice (14). It should be noted that in CPA immunosuppressed mice the increase in cell number is probably reduced by the immunosuppressive, anti-proliferative effect of CPA. It should be noted that in the fungal infection model, the increase in microglia cell number is avoided as last dose of CPA was administered two days prior to the sacrifice, whereas in DSS-CPS treated mice, the last dose of immunosuppressive drug was administered 5 days before the sacrifice and this difference may account for the low but detectable cell proliferation level found in DSS-CPA treated mice.

Although peripheral inflammation exacerbates the shift produced by CPA treatment, DSS-induced colitis did not cause relevant effect on retinal microglia by itself (only a moderate cell proliferation, without increase in CD11b mean intensity or cell heterogeneity), consistent with the fact that no bacterial translocation was observed in these conditions. Anyway, the possibility that extended (chronic) inflammation may cause or enhance retinal microglia activation should not be ruled out, as pro-inflammatory-circulating cytokines generated in response to the inflammatory process could also contribute to retinal microglia activation.

The specific role of microglia in retinal neurodegenerative diseases still remains unknown. The activation of microglia by several stimuli drives to different responses: protective, harmful or with dual effects, and sometimes both effects seem to occur at the same time (1, 41). Our results suggest that immunosuppression, either by immunosuppressive diseases or medical treatments, as well as peripheral inflammation, may contribute to microglia activation, and could worsen the course of retinal dystrophies. Furthermore, our results confirm that invasive candidiasis generated by translocation of fungal cells from the GI tract of colonized and immunosuppressed mice

also affect retinal microglia, as previously described for invasive candidiasis following intravenous injection of fungal cells (14). Hence, non-ocular injuries, as a general immunosuppressive state or a peripheral inflammation, might be underestimated risk factors that could affect the evolution of neurodegenerative disorders. This could be most relevant in elderly patients with a high prevalence of chronic side pathologies. The knowledge of the factors involved in the highly intricate situation that exists in a degenerative pathology should improve the therapeutic options for the affected patients.

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DISCLOSURE

The authors declare no conflict of interests.

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FIGURE LEGENDS

Figure 1. Cell heterogeneity and CD11b expression in retinal microglia of immunosuppressed mice with colitis (A-E). (A) Retinal cells from C57BL/6J untreated mice were labelled with FITC-conjugated anti-CD11b antibody and analyzed by flow cytometry (10^6 cells were analyzed in each assay); CD11b positive cells were gated. (B-E) Contour plots representing FSC against FITC-CD11b fluorescence values of gated cells in a control (B), a DSS treated (C), DSS+CPA-treated (D) and a DSS+CPA-treated (E) mice. Results from a single representative assay are shown. (F-H) Mean area values delimited by external lines in density plots of mouse microglial population analysis (F), mean fluorescence intensity for CD11b (G) and percentage of CD11b positive cells (H) for control mice, and mice treated with DSS, CPA and DSS+CPA. Results are mean values ± SD from at least three mice for each condition.

Figure 2. Confocal immunofluorescence images of retinal microglial cells from an untreated mouse (A-C) and a DSS+CPA-treated mouse (D-F). Immunoreactivity against anti-IBA1 antibody (A, D), anti-MHC class-II RT1B antibody (B, E) or both (C, F) is shown. Arrows indicate the microglia cell soma. Arrowheads point to non-specific immunoreactivity of the secondary antibody to blood vessels. Scale bars 10 μm. Images from CPA-treated mice are similar to the ones obtained with DSS+CPA treatment (not shown).

Figure 3. (A-C) Retinal sections of control (A), DSS+CPA-treated (B) and rd10 mice (C) stained with hematoxylin. (D-F) Retinal sections of control (D), DSS+CPA-treated (C) and rd10 mice (F) immunostained with anti-GFAP antibody. Arrows point to

GFAP-positive Müller cells in the rd10 mouse (F) that were not present in control (D) or DSS+CPA-treated mice. Scale bars: 100 μm (A-C), 40 μm (D-F).

Figure 4. Cell heterogeneity and CD11b expression in retinal microglia of immunosuppressed wild type and TLR2 KO mice colonized by *C. albicans*. (A) Mean area values delimited by external lines in density plots of microglial population analysis (gated as described in Fig. 1) from *C. albicans* colonized wild type (100% relative value) and TLR2 KO mice. (B) Mean fluorescence intensity for CD11b in retinal microglia from *C. albicans* colonized wild type and TLR2 KO mice. (C) Percentage of CD11b positive cells. Results are mean values ± SD from at least three mice for each condition.

Figure 5. TLR2 expression in retinal microglia from control and DSS-CPA treated mice. Retinal cells, from C57BL/6J untreated mice and DSS-CPA treated mice, were labelled with FITC-conjugated anti-CD11b antibody and PE-conjugated anti-TLR2 antibody, and analyzed by flow cytometry (10^6 cells were analyzed in each assay). CD11b positive cells were gated as described in Fig. 1, and mean PE-fluorescence intensity (TLR2-labeling) was measured. Samples labelled with control isotype antibody were used as controls. Results shown are from a pooled sample of retinas from five mice each group.

LIST OF ABBREVIATIONS

CFU	Colony forming units
CPA	Cyclophosphamide
DSS	Dextran sulphate sodium salt
MHCII	Major histocompatibility complex class II

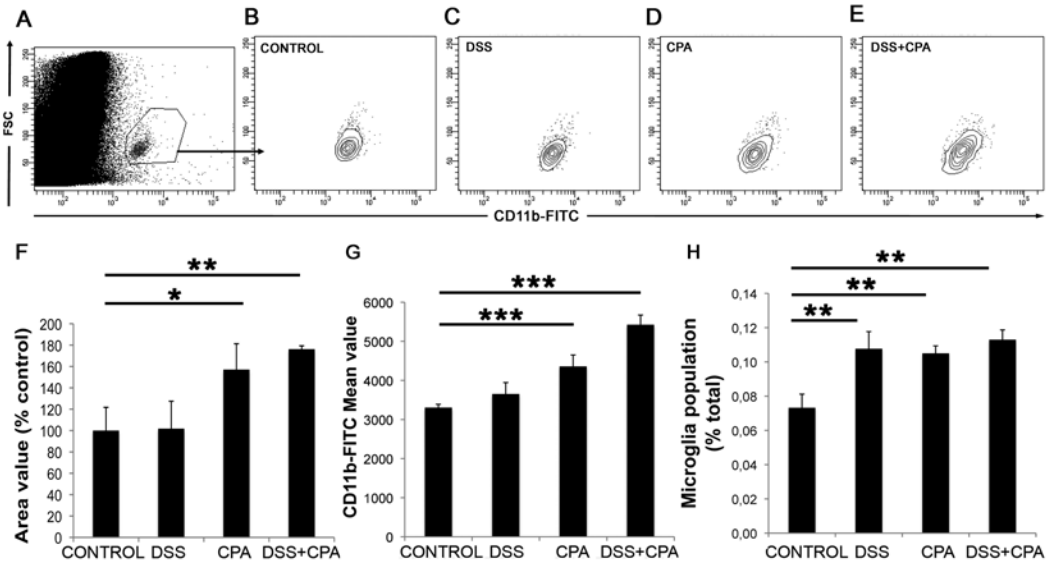

Figure 1

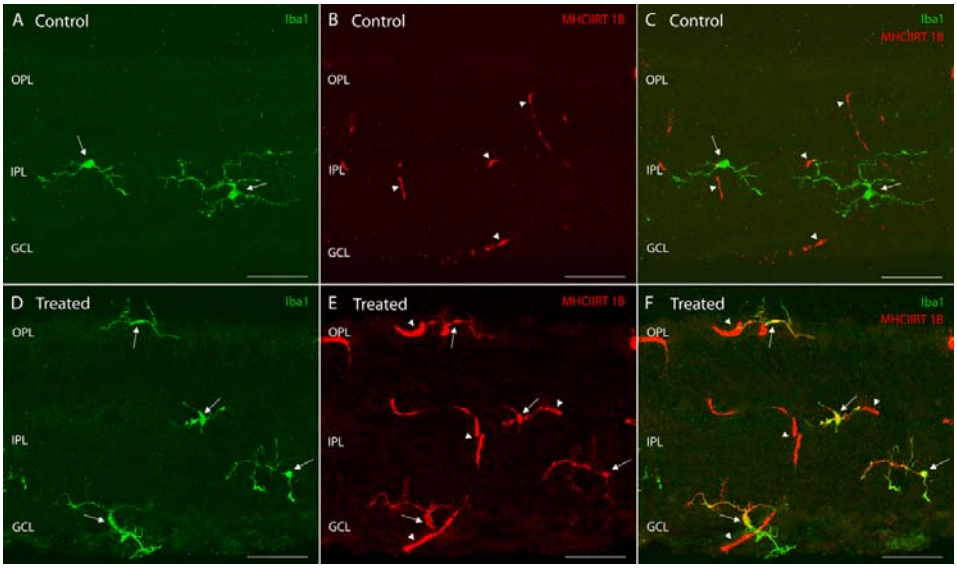

Figure 2

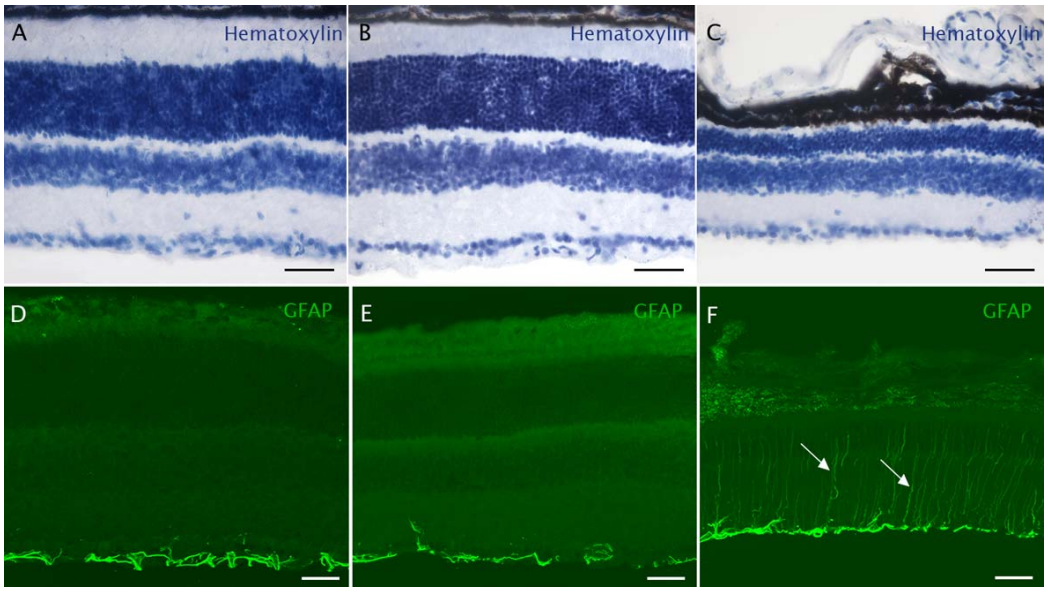

Figure 3

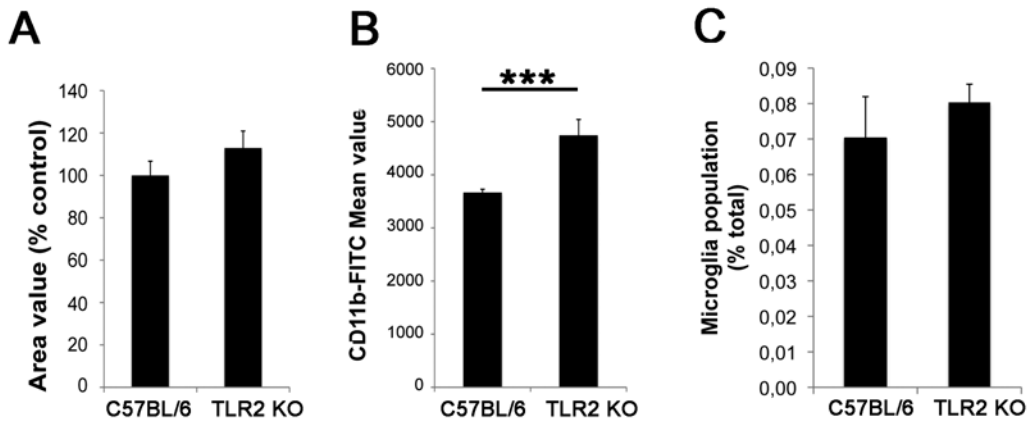

Figure 4

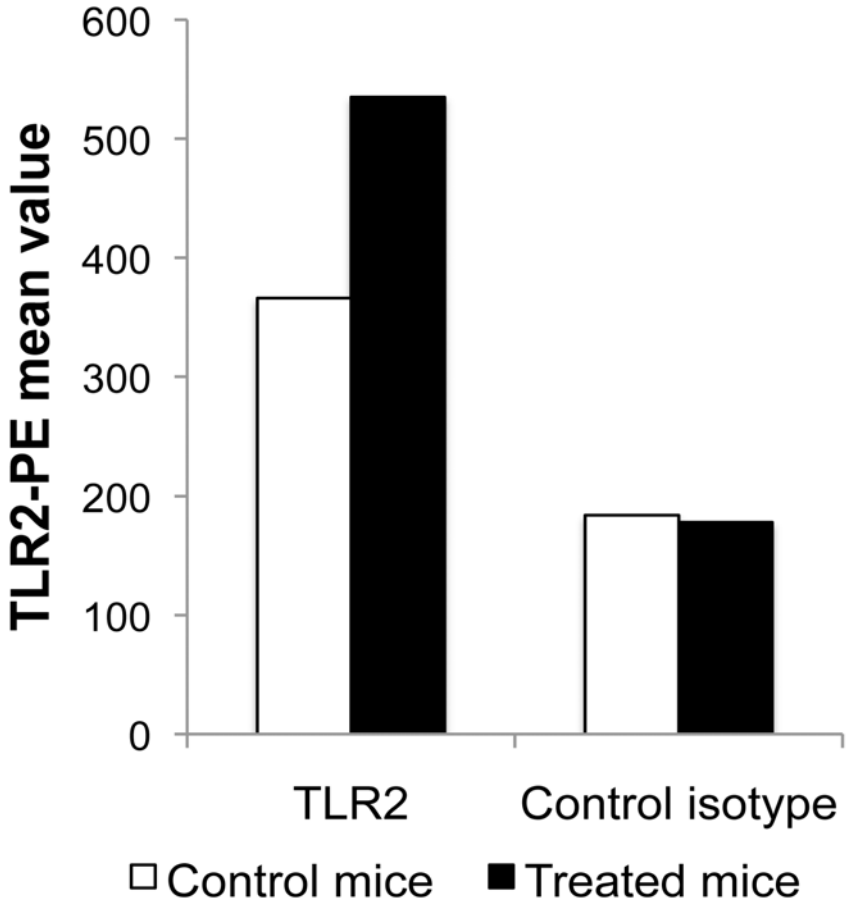

Figure 5